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Seasonal bloom dynamics and ecophysiology of the freshwater sister clade of SAR11 bacteria 'that rule the waves' (LD12)

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Abstract

Alphaproteobacteria are common members of marine bacterioplankton assemblages, but are believed to be rare in lacustrine systems. However, uncultured *Alphaproteobacteria* of the freshwater LD12 lineage form a tight monophyletic sister group with the numerically dominant bacteria in marine epipelagic waters, the SAR11 clade or genus *Pelagibacter*. Comparative rRNA sequence analysis reveals a global occurrence of LD12 bacteria in freshwater systems. The association of genotypic subclades with single study systems moreover suggests a regional diversification. LD12 bacteria exhibit distinct and annually recurring spatio-temporal distribution patterns in prealpine lakes, as assessed by seasonally resolved vertical profiling and high throughput cell counting. During the summer months these ultramicrobacteria can form cell densities in the surface (epilimnetic) water layers that are comparable to those of their marine counterparts (>5×10^8 cells l^{-1}). LD12 bacteria had a pronounced preference for glutamine and glutamate over 7 other amino acids *in situ*, and they exhibited substantially higher uptake of these two substrates (and glycine) than the microbial assemblage in general. In addition, members of LD12 were also able to exploit other monomeric sources of organic carbon such as glucose, fructose, or acetate. LD12 seemed to follow an oligotrophic lifestyle with slow but efficient uptake already at low substrate concentrations. Thus, LD12 bacteria do not only share phenotypic and metabolic traits with *Pelagibacter,* but also appear to thrive in the analogous spatiotemporal niche in freshwaters. The two groups together form one of the rare monophyletic lineages of ultramicrobacteria that have successfully traversed the barrier between marine and freshwater habitats.

Introduction

The freshwater bacterioplankton is believed to be numerically dominated by small *Actinobacteria, Betaproteobacteria* and microbes related to the *Flavo*- and *Sphingobacteriales* (Glöckner *et al.*, 1999, Glöckner *et al.*, 2000, Zwart *et al.*, 2002). By contrast, *Alphaproteobacteria* are regarded to be rare in the pelagic zones of most lacustrine ecosystems (as opposed to marine pelagic habitats), with only very few exceptions (Kirchman *et al.*, 2004, Nishimura & Nagata, 2007). This view is mainly based upon the results of quantitative rRNA based methodology, e.g. of whole cell fluorescence *in situ* hybridization techniques (for review see table VI in Klammer *et al.*, 2002, and Appendix 3 in Nishimura & Nagata, 2007). However, the presumably most abundant microbial species on earth, the marine bacterium *Pelagibacter ubique* and relatives – also known as SAR11-cluster (*Alphaproteobacteria*) (Carlson *et al.*, 2008, Morris *et al.*, 2002, Rappe *et al.*, 2002) – has both a brackish and a freshwater sister group (Bahr *et al.*, 1996, Carlson *et al.*, 2008). Members of the species-like (>98% sequence similarity) freshwater cluster, termed LD12 (Zwart *et al.*, 1998), alpha V (Glöckner *et al.*, 2000), or SAR11 IV (Carlson *et al.*, 2008), are only known from environmental 16S rRNA gene sequences. A ubiquitous occurrence of these bacteria in lakes (Bahr *et al.*, 1996, Zwart *et al.*, 2002) has been suggested, albeit based on a limited number of complete sequences available in public databases. It is unknown if members of the LD12 clade are only an exotic remnant lineage and form small populations in lake water, or if they are as successful and ecologically relevant in freshwater pelagic habitats as their marine counterparts.

The success of SAR11 bacteria in marine waters is attributed to a number of features that render them well adapted to the nutrient and energy limited conditions in offshore habitats such as the photic zone of the Sargasso Sea (Morris *et al.*, 2002).

Cultivated representatives (*Pelagibacter ubique*) are very small (ultramicrobacteria, cell volume < 0.01 μm^3; (Rappe *et al.*, 2002)) with a streamlined genome dominated by transport functions (Giovannoni *et al.*, 2005b, Sowell *et al.*, 2009) which are driven by light dependent proton pumps (proteorhodopsins; (Giovannoni *et al.*, 2005a)). Moreover, they are centrally involved in the remineralization of low molecular weight organic matter such as amino acids or glucose (Alonso & Pernthaler, 2006, Malmstrom *et al.*, 2004), but require exogenous reduced sulfur compounds for growth (Tripp *et al.*, 2008). Several ecotypes within the SAR11 group have been identified and linked to either spatio-temporal habitat preferences (Carlson *et al.*, 2008) or to ocean productivity (Schwalbach *et al.*, 2010).

We investigated the quantitative importance and potential role of bacteria of the LD12 lineage in freshwater habitats by studying i) their occurrence and quantitative importance in several freshwater lakes with different hydrochemical and limnological properties, ii) their seasonal and vertical distribution patterns at high spatio-temporal resolution, and iii) selected metabolic traits, i.e. the incorporation of various low molecular weight organic compounds. Our results suggest that members of the LD12 clade are one of the most abundant ubiquitous lineages of freshwater bacteria whose quantitative importance so far has been largely overlooked. We furthermore could gain first insight into the ecological niche of LD12 bacteria both by identifying their spatiotemporal distribution maxima and by assessing their specific substrate preferences.

Materials and Methods

Sampling

Lake Zurich is a large, deep, prealpine, mesotrophic lake, characterized by persistent annual blooms of the toxic cyanobacterium *Planktothrix rubescens* (Bossard *et al.*, 2001, Van den Wyngaert *et al.*, 2011). Water samples of 53 depth profiles were collected nearby the deepest point of the lake (136 m) in a biweekly interval between January 17th 2008 and December 21st 2009. Core sampling depths were 0, 5, 10, 20, 30, 40, 60, 80, and 100 m, and additional samples were taken from seemingly interesting depths (i.e. depths of chlorophyll *a* maxima or maxima of *P. rubescens*), resulting in a total sample number of 490. Forty ml of water were fixed with formaldehyde (2% final concentration) for flow cytometry, and 5-10 ml were fixed with buffered paraformaldehyde (2% f.c.) for CARD-FISH analyses. Temperature profiles were recorded with an YSI multiprobe.

Piburger See, a small oligo-mesotrophic lake with a maximum depth of 24.6 m is situated in the Austrian Alps and was sampled in a monthly interval between February 2005 and February 2006 (Salcher *et al.*, 2008). Water samples of 3, 9, 18, and 24 m depth were fixed as mentioned above, and unfixed water for autoradiographic assays (see below) was transported to the lab within 2 hours. Water temperature was directly determined during sampling. Thalwiler Waldweiher is a small, shallow (5.5 m maximum depth), eutrophic pond, which was sampled in a monthly interval in a 1 m depth resolution during the ice free period between May 2008 and July 2009. Temperature profiles were recorded with an YSI multiprobe. Lake Constance is a large, deep (250 m), oligotrophic lake connected to Gnadensee (shallow, mesotrophic) and Zellersee (shallow, mesotrophic) (Güde *et al.*, 1998). Lake Constance, Gnadensee, Zeller See, Badsee (small, shallow, eutrophic), and Federsee

(small, shallow, eutrophic) were sampled in July 2009. Samples from 0, 5, 10, 15, 20, and 30 m depth were analyzed from Lake Constance, and surface water samples (Table 1) were taken from the other lakes. Egelsee is a shallow, meromictic, meso-eutrophic lake with a pronounced layer of green and purple sulfur bacteria (*Chlorobium* sp., *Thiopedia* sp., and *Chromatium* sp.) between 6-7 m depth. A depth profile of this lake was acquired in September 2007. Depth profiles of the large, deep, and oligotrophic lakes Lago Maggiore (372 m depth) and Lake Thun (217 m depth) were gained in July 2010 and Gossenköllesee (small, ultraoligotrophic, high mountain lake (Pernthaler *et al.*, 1998)) was sampled in August 2005. Horgener Waldweiher (small, shallow, eutrophic) was sampled in June 2009.

Cloning, sequence analysis of 16S rRNA genes, and probe design

Twohundredfifty ml of unfixed water samples from Lake Zurich from 10 m, 12.5 m, and 15 m depth from October 1st 2007 were filtered onto white polycarbonate membranes and stored frozen until further processing. DNA was extracted with the powerbead soil isolation kit (MOBIO) and 16S rRNA genes were amplified with primers GM3f and GM4r (Muyzer *et al.*, 1995). PCR products were purified with the QIAquick PCR purification kit (QIAGEN), inserted into TOPO vectors (TOPO TA cloning kit for sequencing; Invitrogen) and cloned into competent *E. coli* according to the manufacturer's instructions. After screening of the clones for right-sized inserts, plasmid preparations were done with the QIAprep Spin Miniprep Kit (QIAGEN). The sequencing reactions were accomplished with the primers GM1f (Muyzer *et al.*, 1993) the vector primers M13f and M13r (Messing *et al.*, 1983) and the ABI BigDye chemistry on an ABI 3730 Genetic Analyzer (Applied Biosystems). Partial sequences were assembled with the DNA baser software (HeracleSoftware) and checked for

chimeric origin using Mallard and Pintail (Ashelford *et al.*, 2005). These sequence data have been submitted to the EMBL database under accession No. FN665702-FN665785. Phylogenetic analyses were performed with the ARB software package (Ludwig *et al.*, 2004) using the ARB reference database SILVA SSU100. All sequences were automatically aligned by the SINA web aligner (www.arb-silva.de) and alignments were subsequently manually optimized. Only sequences >1200 nucleotides were used for tree reconstruction, where first a maximum parsimony tree was built. All good quality sequences affiliated with SAR11 (2543) and LD12 (330) were selected for bootstrapped maximum likelihood analyses (100 repetitions) using the RAxML web server (Stamatakis *et al.*, 2005). The obtained trees were manually compared, reduced by 251 almost identical LD12 sequences (mostly from Lake Gatun (Rusch *et al.*, 2007)), and nodes with bootstrap values <50% were collapsed into multifurcations. Probe design for the LD12 cluster was done with the respective ARB tools probe_design and probe_check and obtained probes were also checked for specificity in the ribosomal database project (http://rdp.cme.msu.edu). The resulting probe LD12-121 (5'-CAC AAG GCA GAT TCC CAC AT-3') was tested with different formamide concentrations until stringency was achieved at 35%.

Enumeration of microbes

Total bacterial abundances were counted by an inFlux V-GS cell sorter (Becton Dickinson) equipped with a UV laser (Lightwave Electronics, CY-PS, 60 mW, wavelength of 355 nm), a blue laser (Coherent, Saphire, 200 mW, wavelength of 488 nm), and detectors for 2 scatter and 6 fluorescence channels. All samples were stained with DAPI (4',6-diamidino-2-phenylindole, 1 μg ml^{-1} final concentration), and scatter plots were analyzed with the software FlowJo 7.2.2. (Tree Star).

149 CARD-FISH (fluorescence *in situ* hybridization followed by catalyzed
150 reporter deposition) was carried out as previously described (Sekar *et al.*, 2003) with
151 slight modifications for probe LD12-121, such as longer signal amplification (45 min)
152 and a higher concentration of Alexa488 labeled tyramides (4 µl ml^{-1} amplification
153 buffer). CARD-FISH stained filters were analyzed by fully automated high-
154 throughput microscopy (Zeder & Pernthaler, 2009), where 3 images were recorded:
155 UV excitation for DAPI stained cells, blue excitation for hybridized cells, and green
156 excitation for autofluorescent cells, which otherwise interfere with hybridization
157 signals. All images were analyzed with an image analysis macro (Zeder,
158 unpublished), and interfering autofluorescent cyanobacteria or debris were
159 individually subtracted from hybridized cells. Ten to 36 high quality images were
160 analyzed for each sample. Replicated hybridization of 28 randomly chosen filters
161 resulted in a mean standard deviation of 0.2% of DAPI (8.2% deviation).
162 Cell volume estimations of bacteria hybridized with probes LD12-121 and
163 EUB I-III were done with the image analysis program LUCIA D (http://www.lim.cz)
164 as described in Posch et al. (2009) for 1 sample of Lake Zurich (August 14^{th} 2008, 0
165 m depth). 400-500 cells were measured and significant differences between length,
166 width, and biovolume of bacteria hybridized with LD12 and EUB I-III were identified
167 by paired t-tests computed in SPSS 16.0.
168
169 *Microautoradiography coupled with CARD-FISH (MAR-FISH)*
170 Autoradiography assays were done with the following radioactive substrates (all
171 purchased from Amersham): [^{3}H]-Thymidine (77 Ci $mmol^{-1}$ specific activity), [^{3}H]-
172 Glucose (29 Ci $mmol^{-1}$ s.a.), [^{14}C]-Fructose (0.316 Ci $mmol^{-1}$ s.a.), [^{14}C]-Acetate (56
173 Ci $mmol^{-1}$ s.a.), [^{3}H]-Amino Acids (45 Ci $mmol^{-1}$ s.a.), [^{3}H]-Arginine (49 Ci $mmol^{-1}$

s.a.), [^{3}H]-Lysine (73 Ci mmol^{-1} s.a.), [^{3}H]-Glutamine (53 Ci mmol^{-1} s.a.), [^{3}H]-Glutamate (45 Ci mmol^{-1} s.a.), [^{14}C]-Alanine (0.154 Ci mmol^{-1} s.a.), [^{14}C]-Aspartate (0.207 Ci mmol^{-1} s.a.), [^{14}C]-Serine (0.255 Ci mmol^{-1} s.a.), [^{14}C]-Glycine (0.101 Ci mmol^{-1} s.a.), and [^{3}H]-Leucine (61 Ci mmol^{-1} s.a.), All substrates were added at 10 nmol l^{-1} final concentrations and incubated for 2 h at *in situ* temperature for Lake Zurich samples from 5 m depth (September 16th, 2009), whereas monthly samples from Piburger See were spiked with 5 nmol l^{-1} [^{3}H]-Amino Acids (48 Ci mmol^{-1} s.a.) and incubated for 1 h at *in situ* temperature. Additionally, a 'substrate affinity' experiment was carried out with [^{3}H]-Glutamine (60 Ci mmol^{-1} s.a.), for Lake Zurich samples (5 m depth, September 28th, 2010). Four different concentrations (0.1 nmol l^{-1}, 1 nmol l^{-1}, 10 nmol l^{-1}, and 100 nmol l^{-1}, 120 min incubation each) and 5 different incubation times (15 min, 30 min, 60 min, 120 min, and 240 min, 10 nmol l^{-1} concentration each) were used to gain more information on the affinity of LD12 to the selected tracer and on incorporation speed. Triplicates plus 2 prefixed controls were processed as outlined in Salcher et al. (2008) with the modification that filter sections were glued (1% agarose) onto cover slips with bacteria facing down, carefully peeled off, and cover slips were mounted onto microscopic slides for autoradiography. Therefore, the very small LD12 cells were not covered by silver grains but lying above the photo emulsion. Cover slips were thereafter embedded in a mounting medium containing DAPI and samples were manually evaluated with a Zeiss Microscope with bright field illumination and blue excitation. At least 100 hybridized cells were inspected per sample.

Results and Discussion

Phylogenetic relationship of freshwater LD12 and marine SAR11 bacteria

Bacterial 16S rRNA gene sequences affiliated with LD12 (n = 370) originated from habitats distributed over several continents and from both hemispheres (Fig. 1), supporting the notion of their ubiquitous distribution (Glöckner *et al.*, 2000, Zwart *et al.*, 2002). Sequences from a single lake (Lake Gatun) in the GOS dataset (Rusch *et al.*, 2007) contributed almost two thirds of all LD12 sequences (n = 202). Our own collection of bacterial 16S rRNA gene sequences from the large mesotrophic Lake Zurich, Switzerland, also yielded a surprisingly high proportion of LD12 related genotypes (84 of 442 sequences). Although the majority of LD12 sequences have been obtained from freshwater habitats, some have also been found in estuarine habitats such as the Chesapeake Bay (n = 3) or the Delaware estuary (n = 20).

Phylogenetic analyses gave significant support for the separation of the marine SAR11 and the freshwater LD12 group (90% bootstrap support, Figure 2). The mean distance between the LD12 cluster and a cultivated SAR11 representative, *Pelagibacter ubique* HTCC1062 (88.9%), indicates that the two lineages are different genera. By contrast, the high sequence similarity within the LD12 cluster (mean similarity: 99.5%) suggests that these bacteria might be classified as a single species. Nevertheless, several distinct subclusters within the LD12 lineage were supported by bootstrap values of >50% (Supplementary Figure S1). Most of these subclusters contained sequences that have been obtained from a single habitat (i.e. Wang Yang River, Lake Gatun, or Lake Zurich), and they typically featured rather low branch lengths. However, a more deeply branching subclade of 10 sequences from Lake Zurich (53% bootstrap support) even contained another well-separated subcluster (63% bootstrap support). This microdiversification points to the existence of specific

ecotypes in different lakes and possibly even in a single lake. Distinct ecotypes of the marine SAR11 cluster have been reported to differ in their spatio-temporal distribution patterns in the Sargasso Sea (Carlson *et al.*, 2008).

Occurrence and habitat preference of LD12 bacteria

We identified and quantified LD12 bacteria in several freshwater habitats by CARD-FISH with a newly designed specific rRNA targeted oligonucleotide probe. The available probe that targets the majority of *Alphaproteobacteria* (ALF968, Amann & Fuchs, 2008) is not suitable for the detection of these microbes, as it features several mismatches with genotypes from the LD12 cluster. Therefore, LD12 bacteria have been overlooked in the analysis of freshwater microbial communities by FISH until now.

A high-resolution sampling campaign (n = 490 samples) over two consecutive years unveiled the spatio-temporal population dynamics of LD12 bacteria in Lake Zurich. These bacteria were always present in lake water, but they also exhibited pronounced depth distribution patterns and seasonal fluctuations (Figure 3). The highest numbers were found in the warm epilimnetic water strata during summer (up to 8.0×10^8 cells l^{-1}). Three distinct abundance maxima were discernable in both successive years: (i) shortly after water temperatures reached >16°C (May–June), (ii) during the warmest period at temperatures >22°C (August–September), and (iii) in autumn (November), immediately before or at the onset of the epilimnetic mixis. Only low proportions of LD12 bacteria were present during winter or in deep hypolimnetic water (<5% of all *Bacteria*). Thus, the seasonal population dynamics of freshwater LD12 bacteria are to some extent comparable with those of their marine sister group: Members of the SAR11 clade have been shown to peak during summer stratification

248 in the euphotic zones of various marine habitats such as the oligotrophic North Pacific
249 Ocean (Eiler *et al.*, 2009), the northwestern Sargasso Sea (Carlson *et al.*, 2008), and
250 the Mediterranean Sea (Alonso-Saez *et al.*, 2007).

251 SAR11 bacteria usually account for one quarter of marine bacterioplankton or
252 more, and their abundances range between 0.3–6.3 × 10^8 cells l^{-1} in the North Pacific
253 Ocean (Eiler *et al.*, 2009), 0.2–3.2 × 10^8 cells l^{-1} in the Sargasso Sea (Carlson *et al.*,
254 2008, Morris *et al.*, 2002), and 0.5–3 × 10^8 cells l^{-1} in the Mediterranean (Alonso-Saez
255 *et al.*, 2007). The abundances of LD12 bacteria in the epilimnion of Lake Zurich
256 during summer stratification (2.1 ± 1.2 × 10^8 cells l^{-1}, range 0.3–8.0 × 10^8 cells l^{-1}, n =
257 109) were comparable to those of their marine counterparts in the euphotic zone
258 (mean, 2 × 10^8 cells l^{-1} (Morris *et al.*, 2002)). LD12 cell densities in the lower strata
259 (hypolimnion) of the lake and during winter (0.5 ± 0.3 × 10^8 cells l^{-1}, range 0.05–2.0 ×
260 10^8 cells l^{-1}, n = 381) exceeded typical values for SAR11 in the aphotic zone, e.g.,
261 those in the mesopelagic realms of the Sargasso Sea (0.2 × 10^8 cells l^{-1} (Carlson *et al.*,
262 2008, Morris *et al.*, 2002)).

263 Seasonal population dynamics of LD12 comparable to those in Lake Zurich
264 were also found in surface waters of 2 other systems with contrasting limnological
265 properties, an oligo-mesotrophic mountain lake (Piburger See, Austria) and a
266 eutrophic pond with only 40 days of water renewal time (Thalwiler Waldweiher,
267 Switzerland) (Figure 4A and 4B). Although the sampling resolution in these habitats
268 was lower (monthly), two distinct summer maxima were nevertheless discernable in
269 both cases. Depth profiles from these two lakes, from a large mesotrophic lake (Lake
270 Constance, Germany), and from a shallow, eutrophic lake (Egelsee, Switzerland)
271 furthermore supported the conclusion that LD12 bacteria may generally prefer the
272 upper epilimnetic water layers (Figure 4C–F). Genome analyses of *P. ubique* revealed

273 the presence of a light-driven proton pump (proteorhodopsin (Giovannoni *et al.*,
274 2005a)), and proteorhodopsin genes related to SAR11 have been obtained from
275 several lakes (Atamna-Ismaeel *et al.*, 2008). This suggests that LD12 bacteria might
276 possess such light harvesting pigments, and it could help to explain their high
277 densities in the light penetrated uppermost layers (Supplementary Figures S2 and S3).
278 Moreover, the close relationship between water temperature and the abundances of
279 LD12 bacteria (Figure 5, Pearson's correlation coefficient = 0.81, $p > 0.001$, $n = 621$)
280 also suggests that these microbes in some aspects share habitat preferences with their
281 marine counterparts SAR11 (Alonso-Saez *et al.*, 2007, Carlson *et al.*, 2008, Morris *et*
282 *al.*, 2002).

283 We also assessed the occurrence of LD12 bacteria in the epilimnion of 8 more
284 lakes with different limnological characteristics, ranging from ultra-oligotrophic to
285 eutrophic (Table 1). LD12 bacteria were also abundant in highly productive systems,
286 which is in contrast with the adaptation to nutrient poor habitats of their marine
287 counterparts. Interestingly, LD12 bacteria were extremely rare in samples from a cold,
288 ultra-oligotrophic high mountain lake (Gossenköllesee, Austria) that is usually
289 covered by ice for 7–8 months and features water temperatures around 10°C during
290 summer (Pernthaler *et al.*, 1998).

291 Finally, LD12 and SAR11 bacteria also seem to share a conspicuously small
292 cell size (mean LD12 biovolume, 0.017 μm^3, Table 2) (Nicastro *et al.*, 2006, Rappe *et*
293 *al.*, 2002). The miniature size of *Pelagibacter ubique* has mainly been attributed to an
294 effective adaptation to nutrient limited environments, as their optimized surface-to-
295 volume ratio points to advantages in nutrient and substrate uptake (Giovannoni *et al.*,
296 2005b). In view of the apparent success of LD12 bacteria in eutrophic ponds and
297 lakes, other advantages of the ultramicrobacterial morphotype might also be of

298 relevance, e.g., the protection against size-selective protistan grazing (Pernthaler,
299 2005).

300 It should be noted that the biomass contribution of LD12 might not be as high
301 as its contribution to total cell numbers due to its small cell size. However, most
302 pelagic bacteria in freshwater systems are of very small cell size (i.e. *Actinobacteria*
303 (Jezbera *et al.*, 2009, Salcher *et al.*, 2010), *Polynucleobacter* sp. (Hahn *et al.*, 2009,
304 Hahn *et al.*, 2010, Wu & Hahn, 2006), or *Betaproteobacteria* affiliated with the LD28
305 cluster (M. Salcher, unpublished data)). The fraction of the typically small, so-called
306 low nucleic acid containing bacteria (LNA bacteria) was 77 ± 5 % in Lake Zurich (n =
307 490), 83 ± 8 % in Piburger See (n = 108), and 78 ± 10 % in Thalwiler Waldweiher (n
308 = 126) during the study period. More than 50 % of these LNA bacteria were
309 *Actinobacteria* and LD12 bacteria, while <5 % of cells from these two groups were
310 present in fractions of bacteria with high nucleic acid content (M. Salcher,
311 unpublished data).

312

313 *Physiological traits of LD12 bacteria*

314 Short-time incorporation assays with a tritiated mixture of amino acids over the
315 course of one year in an oligo-mesotrophic lake (Piburger See) revealed high uptake
316 of this important component of the labile dissolved organic matter pool by LD12
317 bacteria (Weiss & Simon, 1999) (Fig. 4A and C). This agrees with reports that their
318 marine relatives can account for 50% of total amino acid assimilation in the North
319 Atlantic (Malmstrom *et al.*, 2004) and around 25% in the Mediterranean, albeit with
320 high seasonal variations (Alonso-Saez & Gasol, 2007). In Piburger See, highest amino
321 acid uptake (up to 80% of all LD12 cells, or $>2 \times 10^8$ active cells l^{-1}) coincided with
322 the highest abundances of LD12 bacteria, i.e. was observed in the epilimnion during

323 the summer months. By contrast, only around 30% of LD12 microbes incorporated
324 the radiolabelled amino acids during winter and in the deeper water layers (mean
325 number of active cells in the hypolimnion: 0.09×10^8 cells l^{-1}). Thus, LD12 cells were
326 most active when phosphorus concentrations were seasonally low (negative
327 correlation between dissolved phosphorus and the proportion of active LD12 bacteria
328 ($r = -0.645$, $p < 0.001$)). Phosphorus can be the growth-limiting factor of freshwater
329 planktonic bacteria (Carlsson & Caron, 2001), and members of the LD12 clade appear
330 to be particularly well-adapted to thrive in nutrient limited situations (Supplementary
331 Figures S2 and S3). Genomic and metaproteomic evidence suggests that their marine
332 sister group may indeed be specialized for phosphorus transport and uptake
333 (Giovannoni *et al.*, 2005b, Sowell *et al.*, 2009). Marine SAR11 bacteria are also
334 centrally involved in phosphate turnover in the sea, e.g., in the nutrient depleted North
335 Atlantic subtropical gyre (Zubkov *et al.*, 2007). Besides phosphorus, also nitrogen is a
336 limiting factor for bacterial growth, especially during summer at times of nitrate and
337 ammonia depletion (Carlsson & Caron, 2001, Morris & Lewis, 1992). We found a
338 significant negative relationship between LD12 cell densities and nitrate
339 (Supplementary Figures S1 and S2) and ammonia concentrations ($r = 0.54$, $p <$
340 0.001).

341 Analyses of the genome of *P. ubique* has revealed that despite its extreme
342 reduction it nevertheless features a surprisingly high number of genes encoding for
343 transport functions (Giovannoni *et al.*, 2005b), in particular also transporters for
344 nitrogenous compounds (e.g. ammonia or amino acids). Several genes encode for
345 ABC transporters with very narrow solute uptake specificity (Hosie & Poole, 2001)
346 like glutamine or glycine/proline transport, others for general L-amino acid transport
347 with very broad solute range, and there are high-affinity transporters for branched

amino acids like leucine (Giovannoni *et al.*, 2005b). Proteomic analyses of *P. ubique* and metaproteomic marine data also point to a dominance of transport functions in SAR11 bacteria (Sowell *et al.*, 2008, Sowell *et al.*, 2009), with up to 67% of all spectra matching transport proteins, especially for polar amino acids (Sowell *et al.*, 2009).

An ecophysiological assay with 14 different radiolabelled low-molecular-weight substrates conducted in Lake Zurich in late summer 2009 (Fig. 6A and B) revealed a pronounced potential preference of LD12 bacteria for glutamate and glutamine (60% and 46% of LD12 cells, respectively). These amino acids were 3.7 and 2.3 times more frequently incorporated by LD12 cells than by the bacterial community on average, with LD12 representing 48% and 30% of total glutamate or glutamine assimilating bacterial cells, respectively. In addition, the proportion of LD12 cells capable of arginine incorporation was also more than twice as high than of other bacterial cells in general, with LD12 representing 30% of total arginine incorporating bacterial cells. These three amino acids (together with proline) belong to the glutamate family, and arginine can also be *de novo* synthesized from glutamate (Philosof *et al.*, 2009). Amino acids of the glutamate family are important as the main nitrogen carriers during the biosynthesis of other amino acids (Lengeler *et al.*, 1999). Glutamate is the universal donor for α-amino groups in amino acids, which accounts for about 80% of the cellular nitrogen, and glutamine transfers another 15% of nitrogen. While marine SAR11 are poised to assimilate inorganic nitrogen (ammonia (Sowell *et al.*, 2009)), it is conceivable that their freshwater relatives cover their cellular nitrogen demand at least partially by a high assimilation of amino acids of the glutamate family (Figure 6B).

372 In additional experiments conducted in autumn 2010, we added ^{3}H-glutamine
373 at 0.1 -100 nmol l^{-1} concentrations (2 h incubation) and incubated samples pulse-
374 labeled with 10 nmol l^{-1} ^{3}H-glutamine for periods of 15 – 240 min. This revealed a
375 typical adaptation of LD12 bacteria to an oligotrophic life style. High proportions of
376 actively incorporating cells were already detected at very low tracer concentrations
377 (Figure 6C): More LD12 bacteria incorporated glutamine at concentrations of only 1
378 nmol l^{-1}, than at concentrations of 100 nmol l^{-1} (67% vs. 61% MAR positive cells).
379 Moerover, while *Bacteria* in general (as hybridized with probe EUB I-III) showed
380 highest tracer uptake at the highest offered concentration (40% MAR positive cells),
381 LD12 bacteria had a distinct incorporation maximum at 10 nmol l^{-1} (76% MAR
382 positive cells), where they constituted 58% of all active *Bacteria*. A similar adaptation
383 to oligotrophic conditions was described for samples from the North Sea, where
384 SAR11 bacteria showed a distinct affinity for glucose at concentrations of 1 nmol l^{-1}
385 (Alonso & Pernthaler, 2006). On the other hand, LD12 bacteria showed slower uptake
386 'kinetics' of radiolabeled glutamine (10 nmol l^{-1}, Figure 6D): *Bacteria* showed a
387 saturation of the numbers of MAR positive cells already after 1 h of incubation (36%
388 MAR positive cells). By contrast LD12 bacteria reached a comparable saturation
389 plateau only after 2 h of incubation (76% MAR positive cells). Thus we draw the
390 prelimninary conclusion that LD12 seem to follow an oligotrophic lifestyle with slow
391 but efficient uptake already at low substrate concentrations.
392 Glycine was also preferably incorporated by LD12 (7.5 fold higher number of
393 MAR-positive cells than the bacterial average, 62% of the total bacterial glycine
394 assimilation, Figure 6A and B). This is in accordance with the observation that *P.*
395 *ubique* is auxotrophic for this amino acid (Tripp *et al.*, 2009). On the other hand, the
396 branched amino acid leucine, which is mostly applied for assessing activity and

biomass synthesis of aquatic bacteria (Kirchman *et al.*, 1985), was significantly selected against by LD12 (12% MAR-positive LD12 cells vs. 41% MAR-positive *Bacteria*, Figure 6A and B). By contrast, marine SAR11 bacteria readily incorporate leucine at frequencies comparable to the community average (Alonso & Pernthaler, 2006, Malmstrom *et al.*, 2004) and the gene encoding its transporter has been identified in their genome (Giovannoni *et al.*, 2005b).

Glucose was incorporated by only approximately one tenth of LD12 cells, accounting for only 5% of the total number of bacteria with glucose uptake. This is also in contrast to the high glucose uptake observed in their marine counterparts (i.e. between 15–50% of total glucose uptake in the Delaware Estuary (Elifantz *et al.*, 2005), Sargasso Sea (Malmstrom *et al.*, 2005), North Sea (Alonso & Pernthaler, 2006), Mediterranean (Alonso-Saez & Gasol, 2007), and Arctic Ocean (Alonso-Saez *et al.*, 2008)). However, *Pelagibacter* strains were found to differ in their ability to utilize glucose: *P. ubique* isolated from the open ocean entirely lack glycolysis related genes (Giovannoni *et al.*, 2005b), whereas alternative glucose oxidation pathways are present in isolates from the Oregon Coast (Schwalbach *et al.*, 2010). Moreover, the total variants in the Entner-Doudoroff operon were highly correlated with chlorophyll *a* concentrations, suggesting diversification into glucose utilizing coastal ecotypes and open ocean ecotypes incapable of glucose oxidation (Schwalbach *et al.*, 2010). Similarly, variants with or without the ability for glucose assimilation might exist within the freshwater LD12 lineage.

Altogether, ultramicrobacteria affiliated with LD12 seem to be one of the most abundant single species of microbes in freshwater lakes with average to high primary productivity (e.g. more than 30% of all *Bacteria* in Lake Zurich). LD12 bacteria mainly thrive in the upper euphotic water layers during summer, and they play an

important role in microbial assimilation of particular low molecular weight organic compounds. Generally, LD12 seem to follow an oligotrophic lifestyle with slow but efficient uptake at low substrate concentrations. They thus share basic features such as habitat preference and ecophysiological traits with their closely related marine sister group SAR11 (Alonso-Saez *et al.*, 2007, Carlson *et al.*, 2008, Eiler *et al.*, 2009, Morris *et al.*, 2002). The two groups together form one of the rare monophyletic lineages of ultramicrobacteria that have successfully traversed the barrier between marine and freshwater habitats (Logares *et al.*, 2010, Urbach *et al.*, 2001).

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Supplementary information is available at the ISME Journal's website.

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Titles and legends to figures

Figure 1: Ubiquitous distribution of LD12 bacteria detected either by rRNA gene sequences (33 locations) or CARD-FISH (13 locations).

Figure 2: Unrooted maximum likelihood tree of 16S rDNA sequences affiliated with SAR11 I-II, SAR11 III, and LD12. The scale bar at the bottom refers to 0.01 % of sequence divergence.

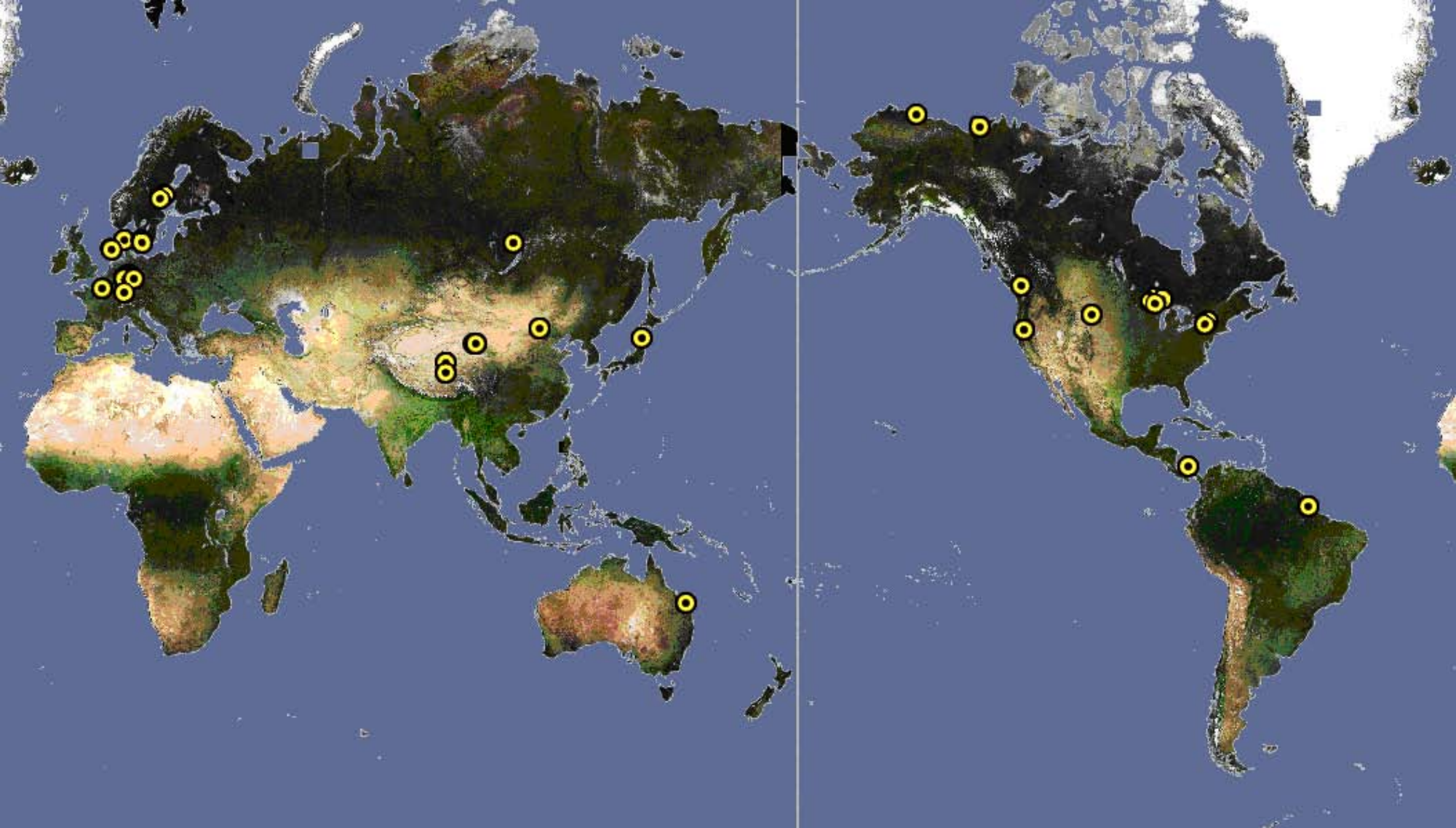
Figure 3: Spatio-temporal distribution of LD12 bacteria in Lake Zurich, Switzerland. The red lines indicate temperature isopleths and open circles refer to sampling points. Mean proportions and standard deviations of LD12 from 0-10 m depths are shown on top and mean seasonal values for all depths on the right (in % of DAPI-stained particles).

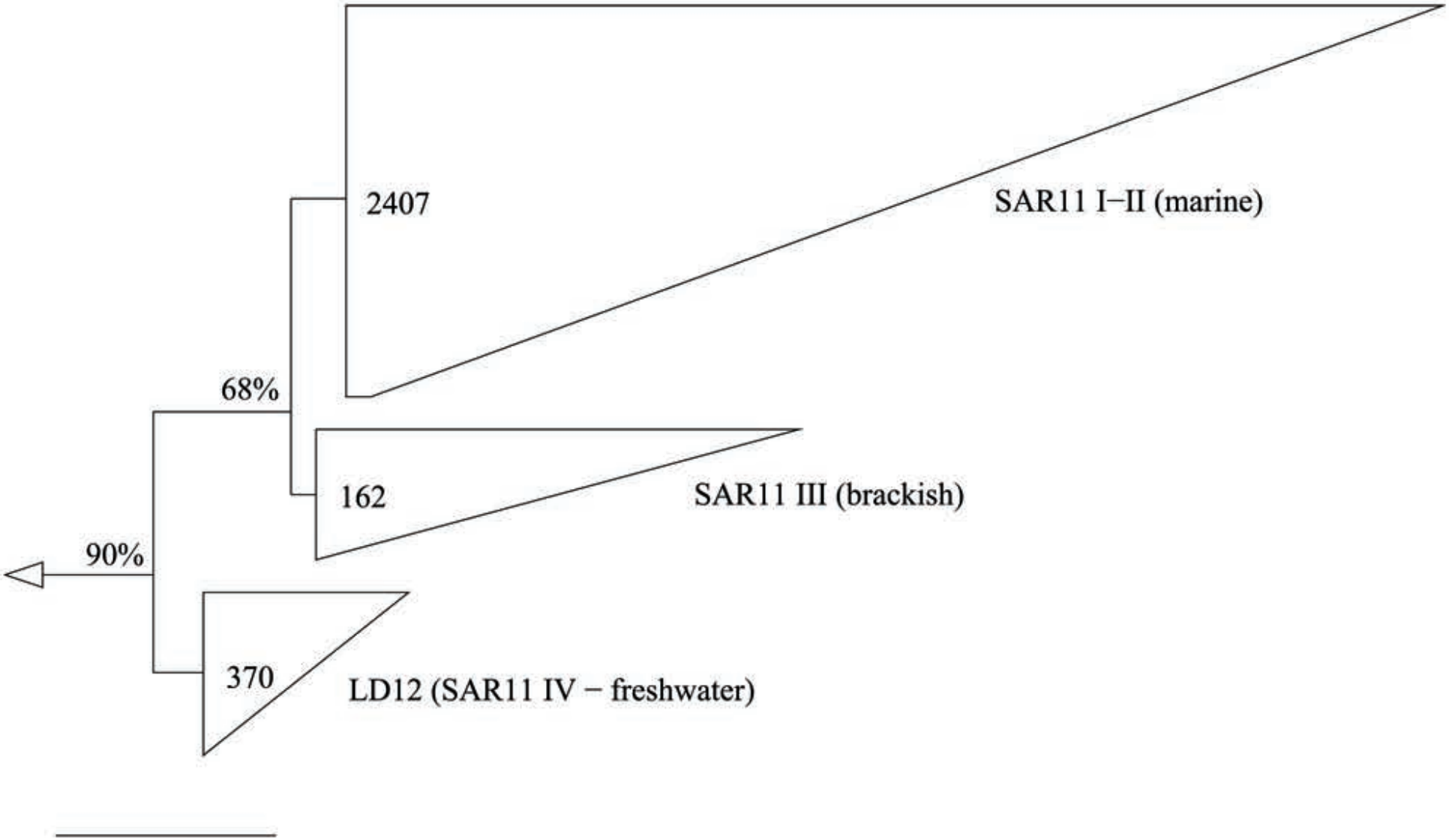
Figure 4: Seasonal and depth distribution of LD12 abundances (white bars) in relation to water temperature (grey area) in temperate lakes. A: Seasonal development of LD12 bacteria in Piburger See, Austria, in 3 m depth and corresponding abundances of LD12 bacteria with active amino acid uptake (grey bars). B: Mean values and standard deviations (n = 3) in Thalwiler Waldweiher, Switzerland, in 0-2 m depth. C: Depth distribution in Piburger See in July 2005 and corresponding abundances of LD12 bacteria with active amino acid uptake, D: depth distribution in Thalwiler Waldweiher in June 2008, E: in Lake Constance, Germany, in July 2009, and F: in Egelsee, Switzerland, in September 2007. n.d., amino acid uptake assay not conducted.

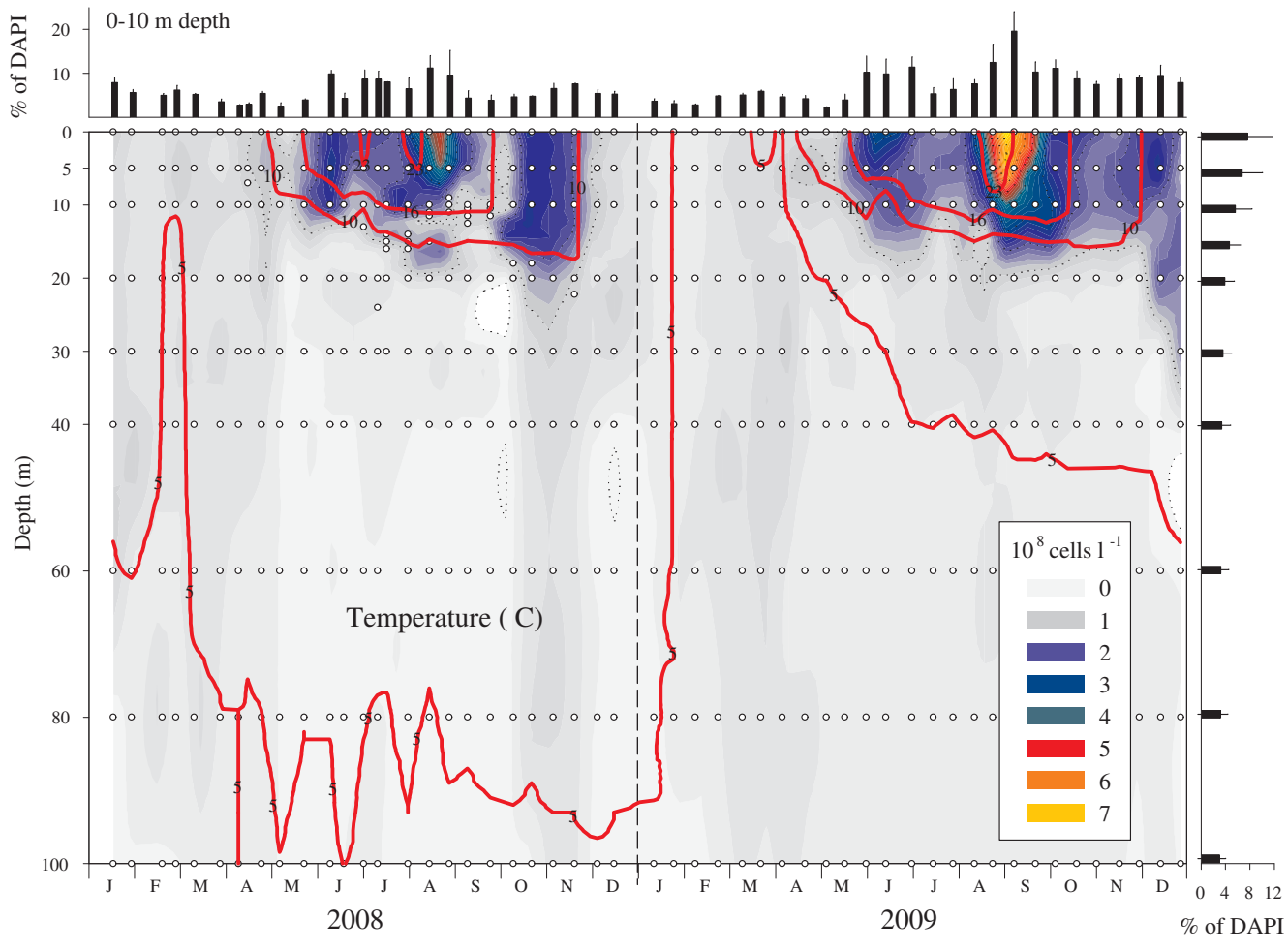
Figure 5: Relationship between LD12 abundance and water temperature in Lake Zurich, Switzerland (blue circles, n = 490), Piburger See, Austria (red triangles, n

= 44), Thalwiler Waldweiher, Austria (green circles, n = 64), and 6 other lakes (orange diamonds, n = 19).

Figure 6, A and B: Ecophysiological MAR-FISH assay with 14 radiolabeled substrates. Means and standard errors (n = 3) of the percentage of all *Bacteria* (A, probe EUB I-III) and LD12 bacteria (B) with active tracer incorporation are shown. AA-Mix; amino acid mixture. C: concentration and D: incubation time dependent incorporation of radiolabelled glutamine of all *Bacteria* and LD12 bacteria. Significant differences between all Bacteria and LD12 bacteria are indicated by underlined x-axis labels. Identical letters in uppercase (EUB) or lowercase (LD12 bacteria) indicate treatments that were statistically indistinguishable.




2407
SAR11 I–II (marine)
68%
162
SAR11 III (brackish)
90%
370
LD12 (SAR11 IV – freshwater)
0.01


0-10 m depth
% of DAPI
20
10
0
Depth (m)
0
5
10
20
30
40
60
80
100
Temperature (C)
10 8 cells l -1
0
1
2
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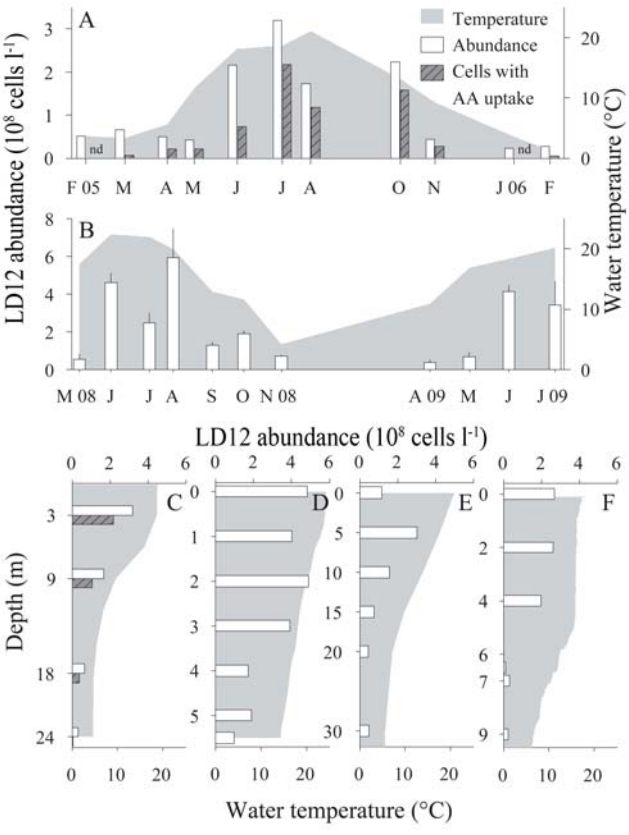
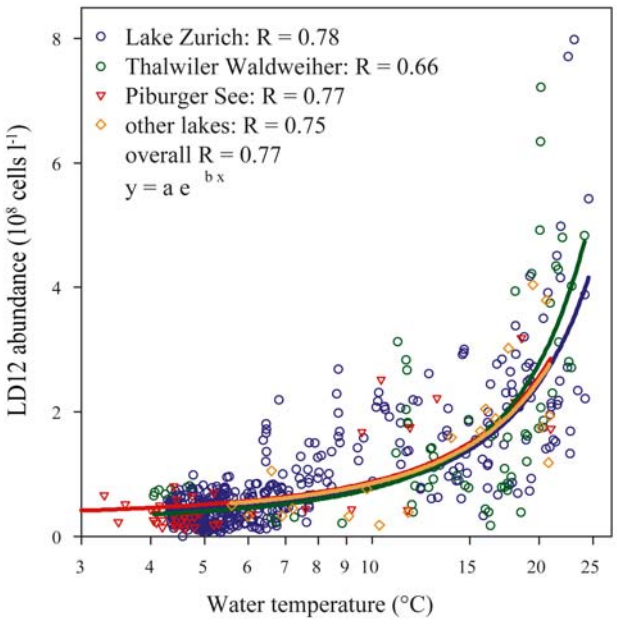
J F M A M J J A S O N D J F M A M J J A S O N D
2008
2009
0 4 8 12
% of DAPI


A
Temperature
Abundance
Cells with
AA uptake
nd
nd
LD12 abundance (10^8 cells l^-1)
Water temperature (°C)
F 05 M A M J J A O N J 06 F
B
M 08 J J A S O N 08 A 09 M J J 09
LD12 abundance (10^8 cells l^-1)
C
D
E
F
Depth (m)
Water temperature (°C)


Lake Zurich: R = 0.78
Thalwiler Waldweiher: R = 0.66
Piburger See: R = 0.77
other lakes: R = 0.75
overall R = 0.77
y = a e b x
LD12 abundance (10^8 cells l^-1)
Water temperature (°C)
0
2
4
6
8
3
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6
7
8
9
10
15
20
25

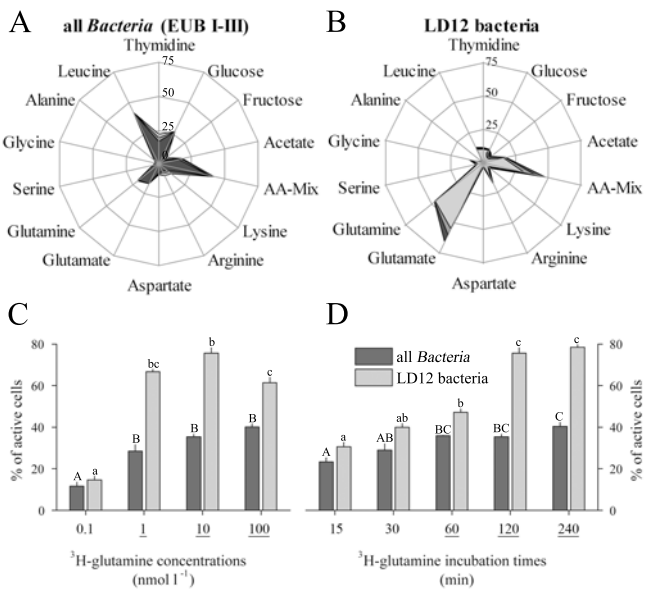
A
all Bacteria (EUB I-III)
B
LD12 bacteria
Thymidine
Glucose
Fructose
Acetate
AA-Mix
Lysine
Arginine
Aspartate
Glutamate
Glutamine
Serine
Glycine
Alanine
Leucine
75
50
25
C
D
% of active cells
all Bacteria
LD12 bacteria
0.1
1
10
100
15
30
60
120
240
3H-glutamine concentrations (nmol l-1)
3H-glutamine incubation times (min)

Table 1: Relative LD12 abundance (% of all *Bacteria*) in different European lakes determined by CARD-FISH with probes LD12-121 and EUB I-III.

Lake	Coordinates	Trophic status	Sampling depths (max depth)	Sampling (n)	Mean % of *Bacteria*	Range
Lake Zurich	47.31 N, 8.58 E	M	0-100 (136)	2 yr (490)	9.2	1.9 – 35.6
Thalwiler Waldweiher	47.28 N, 8.56 E	E	0-5.5 (5.5)	2 yr (64)	14.3	1.9 – 42.9
Piburger See	47.20 N, 10.89 E	O-M	3-24 (24.6)	1 yr (44)	4.7	0.4 – 21.5
Lake Constance	47.65 N, 9.29 E	M	0-30 (252)	Summer (6)	12.8	7.5 – 17.9
Egelsee	47.40 N, 8.36 E	M-E	0-9 (9)	Autumn (6)	6.0	0.4 – 12.7
Lago Maggiore	45.96 N, 8.63 E	O-M	0-350 (372)	Summer (14)	9.1	1.9 – 20.3
Lake Thun	46.69 N, 7.72 E	O	0-213 (217)	Summer (12)	9.7	3.6 – 13.6
Gossenköllesee	47.23 N, 11.01 E	UO	0-8 (8)	Summer (5)	0.8	0.7 – 1.0
Gnadensee	47.70 N, 9.09 E	M	5	Summer (1)	18.2	-
Zeller See	47.72 N, 8.97 E	M	5	Summer (1)	19.4	-
Badsee	47.75 N, 10.0 E	E	1	Summer (1)	21.7	-
Federsee	48.08 N, 9.63 E	E	1	Summer (1)	6.3	-
Horgener Weiher	47.24 N, 8.60 E	E	0.5	Summer (1)	17.8	-

UO = ultraoligotrophic, O = oligotrophic, O-M = oligo-mesotrophic, M = mesotrophic, M-E = meso-eutrophic, E = eutrophic.

Table 2: Size of bacteria affiliated with *Bacteria* (probe EUB I-III), LD12 (probe LD12-121), SAR11, and *Pelagibacter ubique*. Mean values (and ranges) of 400-500 measured cells are given, and significant differences between bacteria hybridized with probes LD12 and EUB are highlighted in bold (paired t-test, df = 461, p > 0.001).

	Length (µm)	Width (µm)	Biovolume (μm^3)	Reference
EUB I-III	0.55 (0.21–4.21)	0.28 (0.13–0.80)	0.038 (0.002–0.569)	This study
LD12-121	**0.38 (0.24–0.69)**	**0.27 (0.19-0.38)**	**0.017 (0.005-0.041)**	This study
Marine SAR11	-	-	0.045 (0.033-0.060)	(Malmstrom *et al.*, 2005)
Pelagibacter ubique	0.4 (0.37-0.89)	0.2 (0.12-0.20)	0.01	(Rappe *et al.*, 2002)
Pelagibacter ubique	-	-	0.025-0.045	(Nicastro *et al.*, 2006)